ARTICLE

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Virus Susceptibility of Chinese Hamster Ovary (CHO) Cells and Detection of Viral Contaminations by Adventitious Agent Testing

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ABSTRACT: Biopharmaceuticals are of increasing importance in the treatment of a variety of diseases. A remaining concern associated with their production is the potential introduction of adventitious agents into their manufacturing process, which may compromise the pathogen safety of a product and potentially cause stock-out situations for important medical supplies. To ensure the safety of biological therapeutics, regulatory guidance requires adventitious agent testing (AAT) of the bulk harvest. AAT is a deliberately promiscuous assay procedure which has been developed to indicate, ideally, the presence of any viral contaminant. One of the most important cell lines used in the production of biopharmaceuticals is Chinese hamster ovary (CHO) cells and while viral infections of CHO cells have occurred, a systematic screen of their virus susceptibility has never been published. We investigated the susceptibility of CHO cells to infection by 14 different viruses, including members of 12 families and representatives or the very species that were implicated in previously reported production cell infections. Based on our results, four different infection outcomes were distinguished, based on the possible combinations of the two factors (i) the induction, or not, of a cytopathic effect and (ii) the ability, or not, to replicate in CHO cells. Our results demonstrate that the current AAT is effective for the detection of viruses which are able to replicate in CHO cells. Due to the restricted virus susceptibility of CHO cells and the routine AAT of bulk harvests, our results provide re-assurance for the very high safety margins of CHO cell-derived biopharmaceuticals.

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KEYWORDS: recombinant products; biopharmaceuticals; virus safety; adventitious agent testing; Chinese hamster ovary (CHO) cells; baby hamster kidney (BHK) cells

Introduction

Biopharmaceuticals such as growth factors, anti-coagulants, therapeutic enzymes and monoclonal antibodies are of increasing importance for public health care. Many of these proteins were initially isolated from tissues or blood of human or other animal origin and had significant viral safety concerns associated with their use. In the 1980s, the first recombinant medicinal products from mammalian cell cultures have become available (for review see Grillberger et al., 2009) and since then, production of therapeutics in cell lines has become prevalent, with more than 165 biopharmaceuticals now approved world-wide (Walsh, 2006). The use of transformed cell lines for the manufacture of therapeutic proteins greatly reduced the risk of human virus transmission, yet other safety concerns arose with the possibility that adventitious agents might be introduced into the manufacturing process through raw materials or personnel. To assure product safety, several measures are in place to minimize the risk of adventitious agent introduction, such as (i) control of the raw material source, (ii) testing of raw materials and the bulk harvest at appropriate stages during the manufacturing process, to confirm absence of adventitious agents (=adventitious agent testing, AAT) and (iii) implementation of virus-inactivation and removal steps.

Chinese hamster ovary (CHO) cells are one of the main cell lines used in the production of recombinant therapeutics (Wurm, 2004; see Grillberger et al., 2009 for examples of proteins produced in CHO cells). The use of this cell line offers several advantages, such as ease of manipulation and a proven safety profile of products from these cells in humans. In addition, a literature survey might suggest that CHO cells are less permissive to infection by viruses than other cell lines used in the production of recombinant proteins, such as baby hamster kidney (BHK) cells (Table I).

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Table I. Overview of previously reported CHO and BHK cell infections by viruses.

Virus family	Species	CHO cells	BHK cells
ss (−) RNA			
Bunyaviridae	Cache Valley virus	Nims (2006), Potts (2008)	—
	Bunyamwera virus	—	Salanueva et al. (2003)
	Northway encephalitis virus	—	McLean et al. (1978)
Orthomyxoviridae	Influenza A/B virus	Kumari et al. (2007), Chu and Whittaker (2004)	Govorkova et al. (1999)
Arenaviridae	Junin virus	—	Ellenberg et al. (2007)
Paramyxoviridae	Parainfluenza virus 1/2/3	Potts (2008)	Nishio et al. (2005), Shimokata et al. (1981), De et al. (1991)
	Simian virus 5	Potts (2008)	He et al. (2002)
	Mumps virus	Wisher (2005), Potts (2008)	McCammon and Riesser (1979)
	Bovine respiratory syncytial virus	Potts (2008)	—
	Sendai virus	—	Nishio et al. (2006)
	Newcastle disease virus	—	Slosaris et al. (1989)
	Pneumonia virus of mice	—	Weir et al. (1988)
Rhabdoviridae	Vesicular stomatitis virus	Potts (2008)	Connor et al. (2006)
	Rabies virus	—	Kallel et al. (2002)
ss (+) RNA			
Coronaviridae	Bovine coronavirus	Francis (2003)	—
	Murine hepatitis virus	—	Matsuyama and Taguchi (2000)
Flaviviridae	Yellow fever virus	—	Patkar and Kuhn (2008)
	Dengue virus	—	Ng et al. (2007)
	West Nile virus	—	Widman et al. (2008), Puig-Basagoiti et al. (2007)
	Tick borne encephalitis virus	—	Goto et al. (2003)
	St. Louis encephalitis virus	—	McLean et al. (1978)
Caliciviridae	Vesivirus 2117	Oehmig et al. (2003), Genzyme (2009)	—
Picornaviridae	Encephalomyocarditis virus	Potts (2008)	Jia et al. (2008)
	Coxsackie virus B-3	Zautner et al. (2006)	—
	Theiler's mouse encephalitis virus	—	Son et al. (2008), Takano-Maruyama et al. (2006)
	Foot and mouth disease virus	—	Vagnozzi et al. (2007), Carrillo et al. (2007), Garcia-Briones et al. (2006)
	Bovine enterovirus	—	Smyth et al. (2002)
	Porcine enterovirus	—	Knowles et al. (1979)
Togaviridae	Semliki Forest virus	Marsh and Bron (1997)	Kiiver et al. (2008), Marsh and Bron (1997)
	Sindbis virus	Potts (2008)	Gorchakov et al. (2008), Sanz et al. (2007)
	Rubella virus	—	Malathi et al. (2001)
	Japanese encephalitis virus	—	Tsai et al. (2007), Su et al. (2002)
	Eastern equine encephalitis virus	—	Petrakova et al. (2005)
	Venezuelan equine encephalitis virus	—	Petrakova et al. (2005)
Arteriviridae	Porcine reproductive and respiratory syndrome virus	—	Shanmukhappa et al. (2007)
Retroviridae	Foamy virus	—	Li et al. (2002)
ds RNA			
Reoviridae	Bluetongue virus	Potts (2008)	De et al. (2005)
	Epizootic hemorrhagic disease virus	Rabenau et al. (1993)	Aradaib et al. (1998)
	Reovirus 1/2/3	Nims (2006), Potts (2008)	Gaillard and Joklik (1985)
	Avian reovirus	—	Chen et al. (2008), Chulu et al. (2007)
	Rotavirus	—	Lopez et al. (2006)
ss DNA			
Parvoviridae	Mice minute virus	Garnick (1996), Nims (2006)	Zoletto (1985), Nettleton and Rweyemamu (1980)
Circoviridae	Porcine circovirus 1	Misinzo et al. (2006)	—
ds DNA			
Adenoviridae	Adenovirus	Condon et al. (2003)	Hosel et al. (2003)
Poxviridae	Modified vaccinia virus 'Ankara'	—	Najera et al. (2006)
Herpesviridae	Pseudorabies virus	Nixdorf et al. (1999)	Slivac et al. (2006)
	Murine gammaherpes 68	—	Gillet et al. (2006)
	Herpes simplex virus 1	—	Conner et al. (2005)
Iridoviridae	Frog virus 3	—	Chinchar et al. (2003)

Viral contaminations of CHO cell-derived bulk harvests are rare, but have occurred. The contaminating viruses were identified as Mice minute virus (Garnick, 1996; Nims, 2006), Reovirus (Nims, 2006), Cache Valley virus (Nims, 2006), Epizootic hemorrhagic disease virus (Rabenau et al., 1993) and most recently, Vesivirus 2117 (Genzyme, 2009). In all of these reported cases, raw materials used in production were suspected as the source of viral contamination (Garnick, 1998; Rabenau et al., 1993; Onions, 2004). Such contamination events usually result in significant interference with production and can lead to complete facility shut down, with a significant risk for stock-out of important medical supplies.

To ensure the safety of recombinant therapeutics, a demonstration of freedom from adventitious viral agents in biopharmaceuticals has become a regulatory requirement (FDA, 1998). AAT procedures have been developed in a deliberately promiscuous fashion to indicate the presence of, ideally, any viral contaminant and involve a broad virus screen on a variety of cell lines, in which cytopathic effect (CPE), hemadsorption (HAD) and hemagglutination (HA) are assayed. In addition, a more specific assay for Mice minute virus is included, as recommended for monoclonal antibody and vaccine production (FDA, 1997; FDA, 2006), in which, for example, CPE and HA are assayed on a susceptible cell line.

Although CHO cells are one of the main cell line used in the production of recombinant therapeutics (Wurm, 2004), no systematic investigation of their virus susceptibility has to date been published. As this information is necessary to understand the safety margins afforded by AAT, we have tested the virus infectability of CHO cells with different viruses, including representatives or the very species involved in previously reported contaminations. The results obtained from this systematic screen confirm the effectiveness of the current AAT system to detect viruses that can replicate in CHO cells.

Materials and Methods

Cells and Viruses

Cells Used for Virus Propagation and Titration

A549 (human epithelial alveolar, American Type Culture Collection [ATCC] no. CCL-185), A9 (mouse fibroblast, CCL-1.4), BT (bovine turbinate, CRL-1390), HeLa (human cervix epithelial, CCL-2) and PK13 (porcine kidney epithelial, CRL-6489) cells were obtained from the ATCC (Rockville, MD.). Vero (African green monkey kidney epithelial) cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK), ECACC no. 84113001.

Cells Used in AAT

CHO (Chinese hamster ovary, CCL-61) and MRC-5 (human lung fibroblast, CCL-171) cells were obtained from the ATCC. 324K (human newborn kidney fibroblast) cells came from American BioResearch Incorporated (lot #981021, Pullman, WA.). The same Vero cells used for virus propagation and titration (see above) were also used in AAT.

Viruses

Viruses were chosen on the basis of literature reports describing their ability to infect CHO cells (Table I). A list of viruses and the cell lines on which they were propagated and titrated is given in Table II. Where the specific virus reported in literature was not available, model viruses were selected, with special emphasis on viruses which might potentially be introduced into the manufacturing process through personnel, for example, Influenza or Parainfluenza virus (Table II).

Inoculation and Incubation of CHO Cells With Virus

CHO cells in 6-well plates were incubated with virus for $2\times 7\pm1$ days, when cell culture supernatant was transferred onto new CHO cells after the first 7 ± 1 days of incubation (Fig. 1). Inoculations with virus were done at a multiplicity of infection (MOI) = 1, that is, approx. 10^5 infectious virus particles per well (with the exception of FPV, for which approx. 10^4 particles/well were used as no higher titer virus stock was available), for 1 h. The inoculum was removed and the cells washed twice with 2 mL of pre-warmed cell culture medium, to remove any un-adsorbed input virus (Fig. 1). The wells were then refilled with 6 mL of CHO medium (Ham's F12 medium supplemented with 10% FCS [Gibco, Invitrogen, Lofer, Austria], L-glutamine [2 mM], non-essential amino acids [1×], sodium pyruvate [1 mM] and Gentamycin sulfate [100 μg/ml]) and plates were incubated for 7 ± 1 days at $36\pm2^{\circ}C$ and $4.5\pm0.5\%$ CO_2, when the CPE on CHO cells was assessed. Consecutively, 2 mL of CHO cell culture supernatant were transferred onto new 6-well plates seeded with CHO cells, filled to a total volume of 6 mL per well with CHO medium and incubated for a further 7 ± 1 days, as before (Fig. 1). The presence of infectious virus was tested by titration at various stages throughout the experiment, on a virus-specific susceptible cell line (Table II) and using a $TCID_{50}$ assay (see below). Samples for titration were taken from (i) the input inoculum, (ii) the second 2 mL washing step, the CHO cell culture supernatant after incubation for (iii) 7 ± 1 days (7d) and for (iv) $2\times 7\pm1$ days (14d), and for (v) the virus inoculum after 14 days of incubation at $36\pm2^{\circ}C$ (hold control, done for selected representatives of enveloped and non-enveloped viruses) (Fig. 1). For each virus, this setup was done in duplicate and repeated at least once.

Infectivity Assays

Samples that potentially contained infectious virus (see above) were titrated by tissue culture infectious dose 50

Table II. Viruses used in this study.

Virus family	Virus/abbreviation	Rationale for inclusion in study	Strain	Origin	Cell line[a]
ss (−) RNA					
Bunyaviridae	Cache Valley virus (CVV)	Previous contamination (Nims, 2006); potential contaminant of cell culture components of bovine origin (e.g., FBS)	Original	ATCC VR-298	A549
Orthomyxoviridae	Influenzavirus A (Fowl plaque virus H7N1, FPV)	Experimental infection reported (Kumari et al., 2007; Chu and Whittaker, 2004); respiratory virus and therefore potential contaminant through manufacturing personnel	A/FPV/ Rostock/45/36	Federal Institute for Risk Evaluation, Berlin, Germany	Vero
Paramyxoviridae	Parainfluenzavirus 3 (PIV-3)	Experimental infection reported (Potts, 2008); respiratory virus and therefore potential contaminant through manufacturing personnel	SF-4	ATCC VR-281	Vero
Rhabdoviridae	Vesicular stomatitis virus (VSV)	Experimental infection reported (Potts, 2008)	PM394	ECACC NCP#401	Vero
ss (+) RNA					
Coronaviridae	Murine hepatitis virus (MHV)	Model for Bovine coronavirus, for which experimental infection has been shown (Francis, 2003)	MHV-JHM	ATCC VR-765	A9
Flaviviridae	Bovine viral diarrhea virus (BVDV)	Potential contaminant of cell culture components of bovine origin (e.g., FBS)	NADL	ATCC VR-1422	BT
Picornaviridae	Encephalomyocarditis virus (EMCV)	Experimental infection reported (Potts, 2008)	EMC	ATCC VR-129B	Vero
	Coxsackievirus B3 (CVB3)	Experimental infection reported (Zautner et al., 2006)	Nancy	ATCC VR-30	Vero
Togaviridae	Ross River virus (RRV)	Model virus for Semliki Forest (Marsh and Bron, 1997) and Sindbis Virus (Potts, 2008), for which experimental infections were reported	na	Queensland University of Technology, Australia	Vero
ds RNA					
Reoviridae	Reovirus 3 (Reo-3)	Previous contamination (Wisher, 2005); model for Epizootic hemorrhagic disease virus, for which contamination has been reported (Rabenau et al., 1993)	Dearing	ATCC VR-824	Vero
ss DNA					
Parvoviridae	Mice minute virus (MMV)	Contamination of cell culture occurred (Garnick, 1996)	Prototype	ATCC VR-1346	A9
	Porcine parvovirus (PPV)	Potential contaminant of cell culture components of porcine origin (e.g., Trypsin)	Tennessee	Biological Research Faculty & Facility #PP951024	PK13
ds DNA					
Adenoviridae	Human adenovirus C, Type 5 (hAdV)	Model for Human adenovirus, for which experimental infection of a CHO cell clone was reported (Condon et al., 2003)	Adenoid 75	ATCC VR-5	HeLa
Herpesviridae	Pseudorabies virus, Suid herpes virus 1 (PRV)	Genetically modified CHO cells are permissive (Nixdorf, 1999)	Kaplan	Fed. Res. Inst. for Viral Dis. of Animals, Tübingen, Germany	Vero

na, not applicable.
[a]Viruses were propagated and titrated on the same cell line.

($TCID_{50}$) assay, that is, eightfold replicates of serial half-log sample dilutions were incubated with cells for 7 days and CPE was assessed microscopically. Virus concentrations were calculated according to the Poisson distribution and expressed as viral load ($\log_{10}$ [$TCID_{50}$]).

Adventitious Agent Testing (AAT)

When results from the CHO cell inoculation and incubation experiments indicated the presence of a replicating virus without/with subtle CPE on CHO cells, the 14d CHO cell

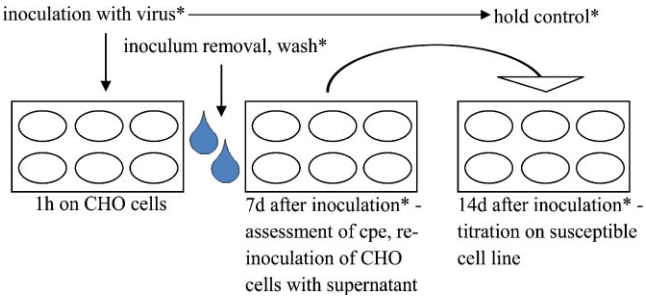

Figure 1. Experimental set-up for the incubation of CHO cells with a panel of selected viruses. CHO cells were inoculated with virus for 1 h. Inoculum was removed, cells were washed with cell culture medium and incubated for 7 days; culture supernatant was then re-inoculated onto fresh CHO cells and incubated for another 7 days; * samples at this stage were titrated on the virus-specific susceptible cell line. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

culture supernatant of the respective virus inoculation was further included in AAT. For this, 0.5 mL of 14d CHO cell culture supernatant was added to each well of 6-well plates containing MRC-5, Vero, CHO or 324K cells and incubated for 1 h. The volume in each well was made up to 6 mL with the respective cell culture medium and plates were incubated for 14 days (MRC-5, Vero and CHO cells) or 21 days (324K cells) at $36 \pm 2^{\circ}C$, when the CPE was assessed and hemadsorption (HAD) and hemagglutination (HA) assays done. For HAD, wells with MRC-5, Vero and CHO cells were covered with erythrocyte suspensions of three different species (human, chicken 0.5% [v/v] and guinea pig 1% [v/v]). Separate plates were incubated at $+2$–$8^{\circ}C$ and $36 \pm 2^{\circ}C$ for 30 min, the cell culture supernatant removed and the cells washed twice with PBS before microscopic inspection for hemadsorption. For HA, supernatants of MRC-5, Vero, CHO and 324K cells were diluted with 0.9% [w/v] NaCl solution in twofold steps. Erythrocyte suspensions (human, chicken 0.5% [v/v] and guinea pig 1.0% [v/v] for MRC-5, Vero and CHO cell supernatants, mouse and guinea pig 1.0% [v/v] for 324K supernatants) were added and separate plates incubated for 35 min at $+2$–$8^{\circ}C$ and $36 \pm 2^{\circ}C$, before hemagglutination was inspected visually. The described AAT is the routine procedure used for the assessment of recombinant protein bulk harvests from CHO cells.

Results and Discussion

The results obtained from the inoculation and incubation of CHO cells with a panel of viruses showed that four different infection outcomes can be distinguished. This differentiation was based on the possible combinations of the two factors (i) induction, or not, of a CPE on CHO cells and (ii) the ability, or not, of the virus to replicate in CHO cells (i.e., the detection of infectious virus on a susceptible cell line, after incubation on CHO cells).

Virus Induced a CPE During CHO Cell Culture and Replicated/Persisted in CHO Cells

After 7 ± 1 days of CHO cell incubation with virus, Human Adenovirus (hAdV), Encephalomyocarditis virus (EMCV), Mice minute virus (MMV) and Reovirus-3 (Reo-3) induced a CPE in CHO cell culture (Fig. 2A, Supplementary Table I). However, the CPE associated with hAdV infection of CHO cells was difficult to assess, as the observed cell changes were subtle and no extensive cell layer destruction occurred. The results obtained from the $TCID_{50}$ assays indicated that these viruses were able to replicate in CHO cells, which was shown by the high infectious virus concentrations obtained after the 7 ± 1 days incubation, as well as after the re-inoculation and second 7 ± 1 days incubation (Fig. 2A, Supplementary Table I). Similar to the CPE results obtained for hAdV, no clear replication was observed for this virus: the load of infectious virus particles did not increase significantly between the second wash and the supernatant after 7 and 14 days of incubation but remained stable at a mean of 3.0–3.6 $\log_{10}$ [$TCID_{50}$] throughout the experiment (Fig. 2A, Supplementary Table I). The hAdV hold control showed that this virus is robust (load of 4.3 $\log_{10}$ [$TCID_{50}$] after 14 days at $36 \pm 2^{\circ}C$, compared to an input of 5.2 $\log_{10}$ [$TCID_{50}$]), suggesting that infectivity detected after 14 days of incubation might be due to residual input virus. As the results obtained with hAdV were difficult to interpret, this virus was included in the routine AAT, to determine whether hAdV can unmistakably be detected in a contaminated sample. During AAT, the supernatant of CHO cells that had been inoculated and incubated with hAdV for 14 days was added to CHO, MRC-5, Vero and 324K cells. A clear CPE was observed on these cell lines after 14 days of incubation, with the exception of CHO cells (Table III). This lack of a CPE on CHO cells during AAT is a further indication that hAdV is not able to reproduce on CHO cells efficiently, an observation that has been reported previously (Condon et al., 2003). Nevertheless, an Adenovirus-permissive CHO cell clone has been described (Condon et al., 2003) and contamination of CHO cells by these viruses cannot be ruled out. In addition to the detection of hAdV through the induction of a CPE on various cell lines during AAT, the presence of hAdV was clearly shown by HA, as a positive result was obtained with the supernatant of MRC-5 cells (Table III).

Overall, the results obtained for these viruses clearly show that the presence of EMCV, MMV and Reo-3 can be reliably detected through the induction of a CPE on CHO cells, whereas a contamination with hAdV is clearly shown during routine AAT.

Virus Induced a CPE During CHO Cell Culture But Did Not Replicate/Replicated Variably in CHO Cells

Ross River virus (RRV), Fowl plague virus (FPV), Cache Valley virus (CVV) and Vesicular stomatitis virus (VSV) rapidly induced a CPE on CHO cells, which became

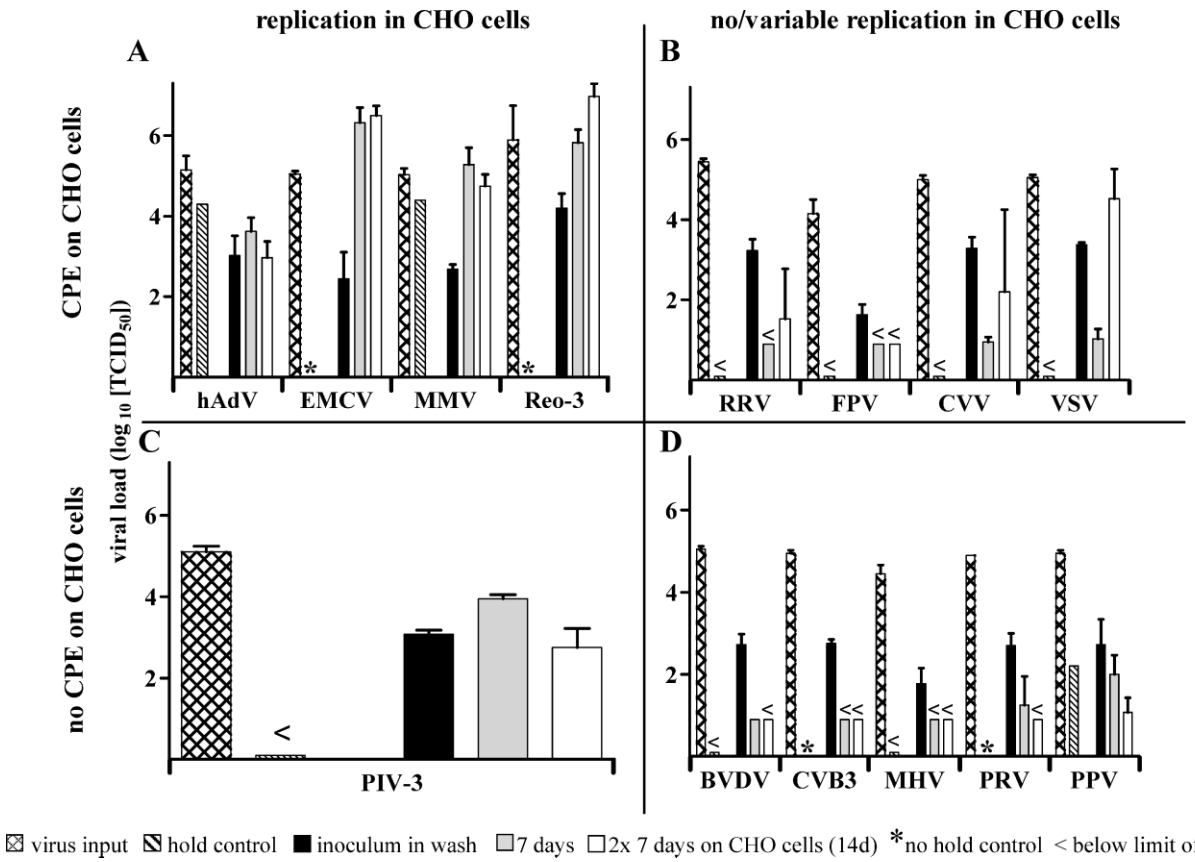

Figure 2. Viruses that (**A** and **B**) induced a cytopathic effect (CPE) or (**C** and **D**) did not induce a CPE on CHO cells. Viruses were (A and C) able to replicate, (B) replicated variably or (D) were not able to replicate on CHO cells. Approximately 10^5 viral particles (▨ virus input, ■ virus present in cell wash after inoculation) were incubated on CHO cells for (▢) 7 days or (□) 14 days or at 36 ± 2°C for 14d without cells (▧ hold control); (*) no hold control. Virus titers were determined by titration of CHO cell supernatants on virus-specific susceptible cell lines (Table II) and expressed as mean viral loads ($\log_{10}$ [$TCID_{50}$]), error bars indicate standard deviation.

Table III. Results of the adventitious agent testing (AAT) in which the culture supernatants of hAdV or PIV-3 infected CHO cells were used as inoculum.

	virus	
Test	hAdV	PIV-3
Cytopathic effect (CPE)		
CHO cells	−	−
MRC-5 cells	+	+
Vero cells	+	+
324K cells	+	+
Hemadsorption (HAD)		
CHO cells	−	+
MRC-5 cells	−	+
Vero cells	−	+
324K cells	na	na
Hemagglutination (HA)		
CHO cells	−	−
MRC-5 cells	+	+
Vero cells	−	+
324K cells	−	+

(−) negative result, (+) positive result, (na) not applicable.

apparent the latest after 6 days of incubation. However, no or rather variable virus replication was observed, characterized by the presence of no or very little infectious virus after the first 7 ± 1 days of CHO cell incubation and a subsequent variable replication during the second 7 ± 1 days of incubation, which resulted, for example, in the detection of virus loads of <0.9 and 5.4 $\log_{10}$ [$TCID_{50}$] for replicates of the same virus (CVV, Fig. 2B, Supplementary Table I). For the Influenza A virus FPV a clear CPE was already observed during the first few days of incubation, yet infectious virus was not detected on the susceptible cell line throughout the course of the experiment. This might have been due to an ability of FPV to initially replicate in CHO cells, which through the induction of a CPE quickly became self-limiting. Subsequently, the thermosensitive progeny virus was inactivated in the CHO supernatant and therefore no infectivity could be detected after re-inoculation on CHO cells and titration on the susceptible cell line. However, this explanation remains speculative and further studies would be required to investigate this observation in more detail.

For RRV, a viral load of 3.4 $\log_{10}$ [$TCID_{50}$] was shown only once (out of four determinations) after re-inoculation

and the second 7 ± 1 days incubation (day 14; Fig. 2B, Supplementary Table I). $TCID_{50}$ titrations of CVV showed the presence of small amounts of infectious virus once (out of six determinations, 1.2 $\log_{10}$ [$TCID_{50}$]) after 7 ± 1 days of virus incubation on CHO cells and twice (out of six determinations) after re-inoculation and the second 7 ± 1 days incubation (4.2 and 5.4 $\log_{10}$ [$TCID_{50}$]; day 14; Fig. 2B, Supplementary Table I). VSV replication in CHO cells was variable and small amounts of infectious virus were detected twice (out of four determinations, 0.9 and 1.4 $\log_{10}$ [$TCID_{50}$]) after the first 7 ± 1 days incubation and a significant titer was obtained in all replicates after re-inoculation and the second 7 ± 1 days of incubation (mean of four determinations: 4.5 $\log_{10}$ [$TCID_{50}$]; day 14, Fig. 2B, Supplementary Table I). These viruses were all rather thermosensitive, as no infectivity was detected in the hold controls after 14 days of incubation at $36 \pm 2^{\circ}C$ (Fig. 2B, Supplementary Table I). The rapidly induced CPE on CHO cells indicated that all of these viruses were initially able to infect and destroy CHO cells, yet no or few replication-competent progeny viruses were released into the supernatant. In the case of CVV and more so VSV, the ability to reproduce on CHO cells appeared to improve after the first 7 ± 1 days incubation, possibly indicating an adaption of the virus to growth in CHO cells, as higher titers were detected more consistently in the 14d CHO cell culture supernatant (Fig. 2B, Supplementary Table I).

In summary, the results obtained for RRV, FPV, CVV, and VSV showed that these viruses rapidly induced a CPE in CHO cells and any contamination of the CHO cell fermenter would therefore be detected.

Virus Did Not Induce a CPE During CHO Cell Culture But Replicated in CHO Cells

Parainfluenza Virus 3 (PIV-3) was included in the study as previous CHO cell infections with this virus, as well as with other members of the *Paramyxoviridae* (e.g., Simian virus 5, Mumps virus and Bovine respiratory syncytial virus) had been reported (Potts, 2008; Wisher, 2005). In this study, PIV-3 was the only virus that did not induce a CPE, yet was able to replicate moderately in CHO cell culture (Fig. 2C, Supplementary Table I). The infectivity detected on the susceptible cell line after the first and second 7 ± 1 days incubation was not due to residual virus inoculum, as the virus was completely inactivated after 14 days at $36 \pm 2^{\circ}C$ (viral load of the hold control <0.1 $\log_{10}$ [$TCID_{50}$]). PIV-3 was therefore included in AAT, to evaluate whether a PIV-3 contamination could be detected during this routine test. During AAT, the supernatant of CHO cells that had been inoculated and incubated with PIV-3 for 14 days was added to CHO, MRC-5, Vero and 324K cells. A clear CPE was observed on these cell lines after 14 days of incubation, with the exception of CHO cells (Table III). In addition, the presence of PIV-3 was clearly shown with HAD (positive result on CHO, MRC-5 and Vero cells) and with HA (positive result by using MRC-5, Vero and 324K cell culture supernatants) assays (Table III).

Overall, the results indicate that Parainfluenza virus contamination of a manufacturing process would be detected by AAT and the effectiveness of the current AAT system to detect an example of a virus that can replicate in CHO cells without an apparent CPE was confirmed.

Virus Did Not Induce a CPE During CHO Cell Culture and Did Not Replicate in CHO Cells

Bovine viral diarrhea virus (BVDV), Coxsackievirus B3 (CVB3), Murine hepatitis virus (MHV), Pseudorabies virus (PRV) and Porcine parvovirus (PPV) did not induce a CPE in CHO cell culture (Fig. 2D, Supplementary Table I). The results obtained from the $TCID_{50}$ assays indicated that these viruses were not able to replicate in CHO cells. No infectious virus was detected throughout the experiment for BVDV, CVB3, and MHV. In the case of PRV and PPV, some residual infectivity was detected after the first 7 ± 1 days incubation (mean of four replicates: 1.3 and 2.0 $\log_{10}$ [$TCID_{50}$], respectively), which was reduced to no detectable infectivity after the second 7 ± 1 days incubation for PRV and to 1.1 $\log_{10}$ [$TCID_{50}$] for PPV (Fig. 2D, Supplementary Table I). This remaining infectivity was most likely due to the robustness of the viruses, as, for example, the viral load of the PPV hold control was 2.2 $\log_{10}$ [$TCID_{50}$] after 14 days at $36 \pm 2^{\circ}C$, compared to a virus input of 5.0 $\log_{10}$ [$TCID_{50}$].

In summary, none of these viruses were able to infect CHO cells and a potential infection of the cell line would therefore not result in virus replication.

Based on published studies and evidence reported at conferences, a comparison of the virus susceptibility of CHO and BHK cell lines indicated that BHK cells are permissive for a broader range of viruses than CHO cells (Table I). For CHO cells, despite their wide use in various biotechnology applications, only a few documented historical contaminations have occurred (Garnick, 1996; Nims, 2006; Rabenau et al., 1993), which strengthens the argument of inherently high safety margins associated with the use of this cell line.

The current study sought to investigate the effectiveness of AAT, through a systematic evaluation of the CHO cell virus susceptibility and included representatives or the very species of viruses that were involved in previously reported infections: MMV, Reo-3 (also as a model for EHDV) and CVV (Garnick, 1996; Nims, 2006; Rabenau et al., 1993), as well as viruses that might be introduced into the manufacturing process as contaminants of raw materials: PPV, PRV, VSV, MHV, BVDV, EMCV, and RRV (as a model for Semliki forest virus) or that might be transmitted by manufacturing personnel: FPV (as a model for Influenzavirus A), PIV-3, hAdV and CVB3. Although experimental infection of CHO cells with Porcine circovirus, a member of the *Circoviridae*, has been shown (Misinzo et al., 2006), this virus was not included in the present screen, as the previously published experiments showed no

productive replication in CHO cells and no substantial safety concerns are therefore expected from this virus. For Vesivirus 2117, a member of the *Caliciviridae* and the virus that was implicated in the most recent widely visible cell culture contamination (Genzyme, 2009), a clearly visible CPE on CHO cells had previously been described (Oehmig, et al., 2003) and the virus is therefore detectable during manufacture and AAT. However, this recent contamination of a manufacturing facility painfully underlines that a remaining risk of such events still exists.

In conclusion, our results demonstrate that viral infections of CHO cells are detectable, either through the induction of a clearly visible CPE, or during routine AAT and we provide evidence that the current AAT system is effective for the detection of viruses that can replicate in CHO cells. Our systematic screen gives evidence for the restricted virus susceptibility of CHO cells (as compared to, e.g., BHK cells), which, in combination with an effective routine AAT of bulk harvests, provides high safety margins for CHO-cell derived biopharmaceuticals.

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